Kidney International, Vol. 46 (1994), pp. 869–876

Autoantibodies against oxidatively-modified LDL in uremic patients undergoing dialysis

ELENA MAGGI, ROBERTO BELLAZZI, ANTONIETTA GAZO, MILFRED SECCIA, and GIORGIO BELLOMO

Department of Internal Medicine and Medical Therapeutics, First Medical Clinic, University of Pavia; Nephrology and Dialysis Unit, USSL 78, Vigevano (PV); and Department of Medical Sciences, 2nd Faculty of Medicine, University of Torino, Novara, Italy

Autoantibodies against oxidatively-modified LDL in uremic patients undergoing dialysis. Target-specific oxidation processes in LDL generate molecular epitopes that are more atherogenic than the native forms and are able to elicit an immunological reaction leading to the formation of anti-oxLDL autoantibodies (oxLDL-Ab) that may participate in the overall process of atherogenesis. Thus, the detection of oxLDLAb, in addition to mirroring the occurrence of *in vivo* LDL oxidation, will give valuable information on the occurrence of this immune response. Plasma oxLDLAb (IgG and IgM) were measured in 72 control subjects (CS) and in 80 patients with chronic renal failure (CRF), undergoing repetitive hemodialysis ($N = 56$) or peritoneal dialysis ($N = 24$), with an ELISA method using native LDL, $CuSO_4$-oxidized LDL (oxLDL) or malondialdehyde-derivatized LDL (MDA-LDL) as antigens. To monitor cross reactivity of the antibodies detected with other oxidatively-modified proteins, human serum albumin (HSA) and MDA-derivatized HSA (MDA-HSA) were also employed as antigens. The antibody titer was calculated as the ratio of antibodies against modified versus native proteins. CRF patients had an antibody ratio significantly higher than CS as concerning anti-oxLDL IgG (1.39 ± 0.36 vs. 1.05 ± 0.3, $P < 0.05$) and IgM (2.15 ± 0.75 vs. 1.43 ± 0.43, $P < 0.01$), and anti-MDA-LDL IgG (3.05 ± 0.74 vs. 2.04 ± 0.42, $P < 0.01$) and IgM (5.55 ± 1.79 vs. 2.9 ± 0.85, $P < 0.01$). The anti-MDA-HSA antibody titer was also higher in CRF patients than in CS (2.49 ± 0.5 vs. 1.46 ± 0.39, $P < 0.01$ for IgG and 2.80 ± 1.03 vs. 1.26 ± 0.43, $P < 0.01$ for IgM). Subclass analysis regarding the type of dialytic treatment revealed that the autoantibody pattern did not differ between CRF patients on hemodialysis and peritoneal dialysis. However, the ratio between anti-MDA-LDL and anti-MDA-HSA (a parameter indicating the specificity of LDL over albumin as the molecule triggering the immunological response) was higher in CRF patients on hemodialysis as compared to peritoneal dialysis (1.34 ± 0.43 vs. 1.12 ± 0.29, $P < 0.05$ for IgG and 2.41 ± 1.22 vs. 1.75 ± 0.78, $P < 0.01$ for IgM). Furthermore, 10% of CRF patients had detectable levels of immune complexes containing oxidized LDL and IgG. These data indicate that CRF patients on dialytic treatment, and particularly on hemodialysis, develop autoantibodies against oxidatively-modified LDL and support the occurrence of an enhanced LDL oxidation *in vivo*.

Atherosclerosis is a multifactorial disease in which multiple cellular and humoral factors are involved [1]. Growing evidence has been obtained indicating that immune reactions, including activation of T lymphocytes [2], aberrant expression of major histocompatibility complex class II antigen by endothelial and smooth muscle cells [3] and immune complex deposition [4], take place during the development and progression of the atherosclerotic lesions. The pathogenetic relevance of these reactions has been validated, for example, by the demonstration that atherosclerotic lesions can be induced in normocholesterolemic rabbits by immunization with heat shock protein 65, a stress protein expressed in high concentration in human atherosclerotic plaques [5]. Moreover, high titers of anti-HSP65 autoantibodies have been detected in patients with carotid atherosclerosis [6].

In a comparable group of patients, Salonen et al [7] have reported that the presence of autoantibodies against LDL derivatized with malondialdehyde (MDA), a product of lipid peroxidation, correlated with the progression of the atherosclerotic disease. These findings were subsequently confirmed by our group in patients undergoing aortocoronary bypass surgery for overt coronary atherosclerosis [8] and in hypertensive patients [9]. All these data prove that antigenic epitopes are generated by oxidative modifications of LDL occurring *in vivo*.

LDL oxidation has been claimed to play a fundamental role in the formation of early atherosclerotic lesions and in their progression [10]. Endothelial and smooth muscle cells as well as monocytes and macrophages are able to trigger the peroxidation of LDL lipids in the subendothelial space leading to the derivatization of the apo B100 molecule with aldehydic products such as MDA and HNE [11]. Oxidative modifications of LDL generate molecular epitopes that provide chemotactic stimuli for monocyte recruitment [12] are more avidly taken up by macrophages, thus forming foam cells [13], and are cytotoxic for endothelial cells [14]. Oxidized lipoproteins (oxLDL) are more atherogenic than their parent forms, and the demonstration that LDL oxidation does actually occur *in vivo* has promoted clinical investigations to ascertain the role played by this process in the progression of atherosclerosis in humans.

Patients with chronic renal failure on dialytic treatment often develop moderate hyperlipidemia and accelerated atherosclerosis, and more than 50% of them die from cardiovascular complications [15, 16]. We have recently reported that LDL isolated from uremic patients are more susceptible to *in vitro* oxidation as compared to LDL from healthy subjects and that an enhanced LDL oxidation does actually takes place *in vivo* [17]. The possibility that an oxidant injury might be implicated in some processes involved in the pathogenesis of atherosclerosis among CRF patients on dialysis was indirectly supported by previous investigations reporting an increased lipid peroxidation [18] and a low

Received for publication January 27, 1994
and in revised form April 13, 1994
Accepted for publication April 14, 1994

Table 1. Clinical and demographic parameters of the CRF patients under study

Parameters	Total uremic patients (N = 80)	Patients on hemodialysis (N = 56)	Patients on peritoneal dialysis (N = 24)
Age *years*	60.7 ± 14.3	61.5 ± 13.6	58.9 ± 16.1
Sex	40 F, 40 M	26 F, 30 M	14 F, 10 M
BMI	24.8 ± 4.8	23.5 ± 3	26.9 ± 5.1
Duration of dialytic treatment *months*	50.5 ± 39.3	54.1 ± 43.4	41.9 ± 26.1
Total cholesterol *mg/dl*	201 ± 52.9	190.2 ± 47.1	227 ± 57
LDL cholesterol *mg/dl*	118 ± 46	109 ± 40	140 ± 52
HDL cholesterol *mg/dl*	43.1 ± 14.3	43 ± 15.3	43 ± 11.8
Triglycerides *mg/dl*	200 ± 92.8	190 ± 80	221 ± 114

glutathione peroxidase activity and selenium concentration [19] during hemodialysis.

In this work we investigated the presence of autoantibodies against oxidatively-modified LDL and oxLDL-containing immune complexes in plasma of CRF patients on dialytic treatment. Data are reported demonstrating the occurrence of an immune response to oxLDL and MDA-modified LDL in uremic patients. The detection of autoantibodies also against MDA-modified HSA indicates that MDA-derivatization may represent the molecular epitope involved. Moreover, the evaluation of the immune response specificity for MDA-LDL over MDA-HSA suggests that immune stimulation by oxLDL is more strictly associated with hemodialysis as compared to peritoneal dialysis.

Methods

Patients

Eighty unselected uremic patients have been investigated: 56 were on repetitive hemodialysis, three times a week with an HCO_3^- dialysis using hemophane (N = 25), cuprophane (N = 10), polyacrylonitrile (N = 19) and cellulose acetate (N = 2) membranes, and 24 were on conventional CAPD treatment (Table 1). They were compared with 72 control subjects matched for age and sex. None of the control subjects had clinically-evident signs of atherosclerotic diseases, hypertension, diabetes mellitus, dislipidemia and renal failure. The majority of the patients investigated (55%) had a post-inflammatory chronic renal failure (glomerulonephritis, pyelonephritis, LES) (Table 2). Diabetes mellitus was present in 12.5% and hypertension was present in 55% of the patients and was clinically well-controlled with conventional hypoglycemic and anti-hypertensive therapy. The mean duration of the dialytic treatment was 54.1 ± 43.4 (range 6 to 190) and 41.9 ± 26.1 (range 7 to 110) months for CRF patients on hemodialysis and peritoneal dialysis, respectively. The adequacy of the dialytic treatment was evaluated using the Kt/V (urea) [20] that ranged from 1 to 1.2 for hemodialytic patients and from 1.6 to 1.8 (weekly) for CAPD patients. The Kt/V was calculated using a commercially available computer program. Each patient was scored on the basis of previous myocardial infarction(s), cerebral transitory or persistent ischemic attack(s), symptomatic ischemic cardiomyopathy and peripheral vasculopathies. From each patient venous blood was taken before the first dialysis session of the week after overnight fasting, in polypropylene tubes containing K-EDTA (final concentration 1 mg EDTA/ml blood), and plasma was collected after centrifugation and stored at −20°C until use.

Table 2. Primary reasons of renal diseases which led to dialytic treatment

Diseases	% Incidence
Polycistic renal disease	11.6
Nephroangiosclerosis	17.9
Chronic glomerulonephritis	15.6
MP glomerulonephritis	2.5
Focal glomerulonephritis	2.5
Membranous glomerulonephritis	1.3
SLE	1.3
Pyelonephritis	10.3
Nephrolithiasis	7.7
IgA nephropathy	2.5
Type I diabetes mellitus	6.4
Type II diabetes mellitus	2.5
Toxic nephropathy	2.5
Uratic nephropathy	1.3
Obstructive or reflux nephropathy	3.7
Unspecified pathology	15.1

LDL preparation

Venous blood was taken from normal volunteers, after overnight fasting, in polypropilene tubes containing K-EDTA (final concentration 1 mg EDTA/ml blood), and plasma was collected after centrifugation. The LDL fraction was isolated from the whole plasma by ultracentrifugation through a KBr discontinuous gradient and collected as the fraction floating at a density of 1.019 to 1.063 g/ml.

Antigen preparation

Non-modified LDL and human serum albumin. Native LDL, obtained after plasma ultracentrifugation, were kept in saline phosphate (10 mM) buffer, pH 7.2 containing EDTA (1 mg/ml) and immediately used to coat ELISA plates. Human serum albumin was purchased as a 20% solution (wt/vol) in saline phosphate buffer from Pasteur Merieux SV and diluted in the same buffer containing EDTA (1 mg/ml).

Oxidized LDL. EDTA was removed from the LDL fraction by rapid filtration through disposable desalting columns Econo-Pac 10 DG (Bio-Rad) and LDL were resuspended in oxygen-saturated phosphate-buffered saline (10 mM Pi, pH 7.2) at a concentration of 0.25 mg LDL mass/ml buffer (50 μg LDL protein/ml = 0.1 μM). LDL oxidation was then triggered by the addition of 2 μM $CuSO_4$ and continuously monitored spectrophotometrically at 234 nm, to follow the formation of conjugated dienes as described by Esterbauer et al [21]. After 18 hours the oxidation process was stopped by the addition of EDTA (1 mg/ml).

MDA-LDL and MDA-HSA. Freshly-isolated LDL (2 mg/ml) or human serum albumin (2 mg/ml) were incubated for three hours at 37°C with 0.5 M MDA, obtained by acid hydrolysis of MDA-bisdimethyl-acetal. Unbound MDA was then removed by rapid filtration through disposable desalting columns Econo-Pac 10 DG (Bio-Rad). Under these conditions a large fraction of the ε-amino group in lysine residues was derivatized.

Evaluation of MDA- and HNE-adducts in modified proteins

The presence of MDA- and HNE-adducts in modified proteins was detected using scanning fluorescence spectroscopy. Native or modified LDL (1 mg/ml) or HSA (1 mg/ml) were resuspended in 10 mM phosphate buffer and analyzed using a Perkin-Elmer LS-5B

Table 3. Adduct formation upon incubation of native LDL and human serum albumin with malondialdehyde

Conditions	Fluorescence arbitrary units (400 nm excitation/470 nm emission)
Native LDL	22.1 ± 1.8
+ 0.25 M malondialdehyde	93.4 ± 5.6
Human serum albumin	2.1 ± 0.3
+ 0.25 M malondialdehyde	107 ± 7.7

Human serum albumin (1 mg/ml) and isolated human LDL (1 mg mass/ml) were incubated without or with 0.25 M malondialdehyde for 3 hours at 37°C in 10 mM phosphate buffer containing 1 mg/ml EDTA, pH 7.4. At the end of the incubation the samples were rapidly filtered through desalting columns in order to remove unbound malondialdehyde and then processed for fluorescence measurements as described in **Methods.**

Table 4. Formation of 4-OH-nonenal- and malondialdehyde-LDL adducts in oxidized human low-density lipoproteins

Fluorescence wavelengths	Native LDL	Cu^{++}-oxidized LDL
	Fluorescence arbitrary units	
360 nm excitation/430 nm emission (HNE-LDL adduct)	12.5 ± 2.3	35.4 ± 5.4[a]
400 nm excitation/470 nm emission (MDA-LDL adduct)	22.1 ± 1.8	38.8 ± 4.4[a]

Isolated human LDL (1 mg mass/ml) were incubated without or with 2 μM $CuSO_4$ for 18 hours at 30°C in 10 mM phosphate buffer, pH 7.4 and then processed for fluorescence measurements as described in **Methods.**
[a] Significantly different from native LDL, $P < 0.01$

Spectrofluorometer. The quantitation of the fluorescence intensity at 360 nm excitation/430 nm emission was taken as an indirect measure of the HNE-protein adduct and at 400 nm excitation/470 nm emission as an indirect measure of the MDA-protein adduct [22].

Measure of anti-oxidized LDL and anti-MDA-modified LDL and serum albumin autoantibodies

The quantitation of the different autoantibodies was performed using an ELISA method. Disposable, 96-well polystyrene plates (Corning) were employed. Antigens for this assay included native LDL and underivatized human serum albumin (protected against oxidation by EDTA), oxidized LDL (obtained after extensive oxidation with 2 μM $CuSO_4$), and LDL and human serum albumin, derivatized with MDA as described above. Each well was coated with 10 μg antigen in PBS for four hours. The remaining binding sites were then blocked using 3% fetal bovine serum in PBS (coating buffer) for two hours at 37°C.

In the present study, 1:11 dilution of plasma from each subject was prepared and 220 μl were added in duplicate to wells coated with native and modified proteins. After incubation at 37°C for two hours, wells were decanted and washed four times before an appropriate peroxidase-conjugated antibody specific for IgG or IgM (diluted 1:2000) was added. After one hour incubation at 37°C and extensive washing, the peroxidase activity was developed using phenylenediamine dihydrochloride and H_2O_2 as revealing reagents. The absorbance was measured at 492 nm in an automatic microplate reader. To calculate antibody titers the ratio between the spectrophotometric readings of anti-modified and anti-native antigen wells was used. Using this approach, the spectrophotometric readings of anti-native antigen wells represented the corresponding blank of anti-modified antigen wells and minimized the possible detection of false positive values due to cross reactivity with both epitopes.

Measure of immune complexes containing oxidized LDL

The quantitation of immune complexes containing oxidized LDL was performed using an ELISA method. Disposable, 96-well polystyrene plates (Corning) were coated with an anti-copper-oxidized human LDL serum developed in rabbit (diluted 1:2000; provided by Prof. Hermann Esterbauer). After coating for four hours at 37°C the remaining binding sites were blocked using 3% fetal bovine serum in PBS (coating buffer) for two hours at 37°C. The rationale behind this assay is that immobilized anti-oxLDL antibodies (which coat the ELISA plate) bind both free and conjugated oxidized LDL (immune complexes) present in diluted plasma. The latter (oxidized LDL/anti-oxidized LDL complex) can be subsequently detected using peroxidase-conjugated anti-IgG or IgM secondary antibody.

In the present study, 1:20 dilution of plasma from each subject was prepared and 220 μl were added in duplicate to wells coated with native and modified proteins. After two hours incubation at 37°C, wells were decanted and washed four times before an appropriate peroxidase-conjugated antibody specific for IgG or IgM (diluted 1:2000) was added. After one hour incubation at 37°C and extensive washing, the peroxidase activity was developed using phenylenediamine dihydrochloride and H_2O_2 as revealing reagents. The absorbance was measured at 492 nm in an automatic microplate reader. Absorbances higher that 1.000 (for a 1:20 dilution of the plasma) were considered positive.

Statistical analysis

All data were statistically analyzed with Student's *t*-test and linear regression analysis using the Micro-Cal Origin and CSS:Statistica programs for personal computers. Results are expressed as mean ± SD.

Results

Antigen characterization: Formation of HNE-LDL and MDA-LDL adducts during LDL oxidation

To obtain the formation of MDA-protein adducts, LDL or human serum albumin, protected from spontaneous oxidation by EDTA, were incubated with MDA. As shown in Table 3, this treatment resulted in a substantial increase of the fluorescence intensity at 400 nm excitation/470 nm emission, indicating the occurrence of derivatization of the ε-amino group in the lysine residues with MDA. Using a similar protocol Palinski et al obtained a derivatization of about 77% of the free amino groups in LDL [23]. Moreover, long-term incubation of isolated human LDL with $CuSO_4$ caused a significant increase of the fluorescence intensity at 360 nm excitation/430 nm emission and 400 nm excitation/470 nm emission, indicating the derivatization of Apo B100 with HNE and MDA occurring during the peroxidation of LDL lipids triggered by Cu^{++} (Table 4), as previously reported [23, 24]. These results indicate that: (i) incubation with MDA actually generates MDA-derivatized LDL and HSA, and (ii) at least two molecular epitopes (namely MDA-LDL and HNE-LDL)

Table 5. Anti-oxidized LDL, anti-malondialdehyde-modified LDL and albumin autoantibodies in plasma of CRF patients

Parameters	Control subjects (N = 72)	Uremic patients (N = 80)
Anti-oxLDL/natLDL IgG ratio	1.05 ± 0.3	1.39 ± 0.36[a]
Anti-MDA-LDL/natLDL IgG ratio	2.04 ± 0.42	3.05 ± 0.74[b]
Anti-MDA-HSA/HSA IgG ratio	1.46 ± 0.39	2.49 ± 0.5[b]
Anti-oxLDL/natLDL IgM ratio	1.43 ± 0.43	2.15 ± 0.75[b]
Anti-MDA-LDL/natLDL IgM ratio	2.9 ± 0.85	5.55 ± 1.79[b]
Anti-MDA-HSA/HSA IgM ratio	1.26 ± 0.43	2.8 ± 1.03[b]

[a] Statistically different from control subjects ($P < 0.05$)
[b] Statistically different from control subjects ($P < 0.01$)

are present in Cu^{++}-oxidized LDL used as antigens in the present study.

Presence of autoantibodies against oxLDL, MDA-LDL and MDA-HSA in uremic patients

As reported in Table 5, CRF patients had an antibody ratio significantly higher than CS regarding anti-oxLDL IgG (1.39 ± 0.36 vs. 1.05 ± 0.3, $P < 0.05$) and IgM (2.15 ± 0.75 vs. 1.43 ± 0.43, $P < 0.01$), and anti-MDA-LDL IgG (3.05 ± 0.74 vs. 2.04 ± 0.42, $P < 0.01$) and IgM (5.55 ± 1.79 vs. 2.9 ± 0.85, $P < 0.01$). No significant difference, however, was detected between patients on peritoneal dialysis and hemodialysis, or, among the latter group, between subgroups using different dialysis filter types. A further analysis of the data obtained (Figs. 1 and 2) revealed a progressive shift of the frequency distribution curve to the right, with a large proportion of the antibody titer detected in CRF patients located above the upper limit of the distribution obtained in control subjects.

Competitive immunoassay studies have revealed that monoclonal and polyclonal anti-MDA-LDL antibodies can cross react with other substrates containing the MDA-lysine adduct [23]. For these reasons we used MDA-modified human serum albumin as antigen to detect the presence of autoantibodies in plasma of CRF patients. As reported in Table 5, the anti-MDA-HSA antibody titer was also higher in CRF patients than in CS (2.49 ± 0.5 vs. 1.46 ± 0.39, $P < 0.01$ for IgG and 2.80 ± 1.03 vs. 1.26 ± 0.43, $P < 0.01$ for IgM). Again, no significant difference was detected between CRF patients on peritoneal dialysis and hemodialysis.

The presence of autoantibodies against two structurally-unrelated proteins, such as LDL and HSA, but which have been derivatized with MDA suggests that the adduct MDA-lysine could be the antigenic determinant. This conclusion is supported by the demonstration of a strict correlation (r = 0.599, $P < 0.001$) between the spectrophotometric readings of anti-MDA-LDL and anti-MDA-HSA IgG (Fig. 3A). Values obtained in control subjects were clustered in the lower left corner of the graph and were encompassed by two lines that delineate four different fields. Interestingly, most of the CRF patients were located in the upper left field, indicating that the antibodies present in their plasma reacted preferentially with MDA-LDL as compared to MDA-HSA. A significant (r = 0.660, $P < 0.001$) correlation was also detected for anti-MDA-LDL and anti-MDA-HSA IgM (Fig. 3B). However, in this case, the values for CRF patients were distributed in the two upper fields, indicating that, at least in 50% of the patients, the antibodies detected recognized MDA-LDL and

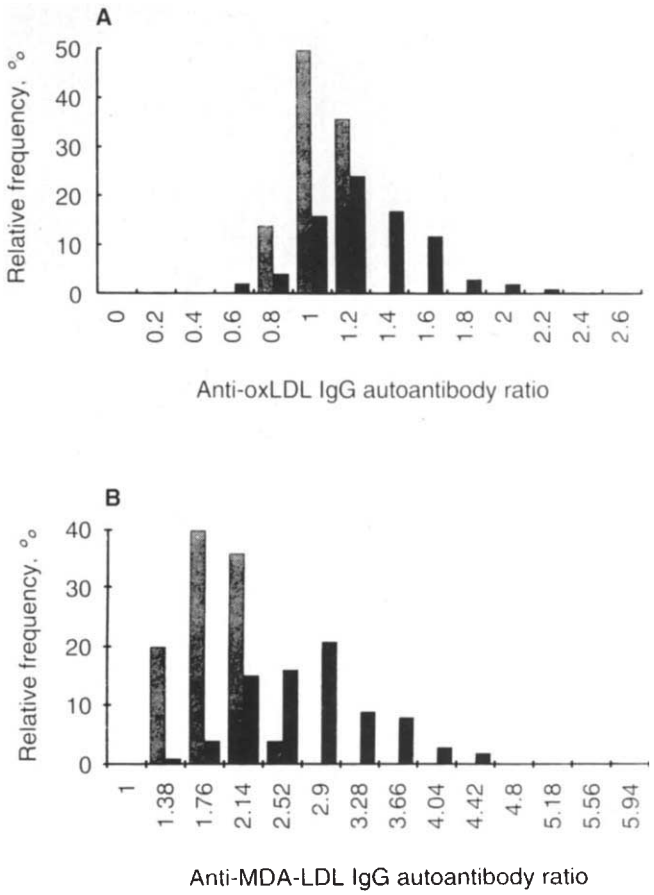

Fig. 1. *Frequency distribution profile of anti-oxLDL and MDA-LDL IgG autoantibodies in control subjects (▨) and CRF patients (■).*

MDA-HSA equally well. As reported above, the derivatization of Apo B100 with MDA and other aldehydic products takes place during *in vitro* oxidation of LDL. Thus, it can be conceivably predicted that a correlation should also exist between anti-MDA-LDL (or anti-MDA-HSA) and anti-oxLDL autoantibodies, and, as reported in Figure 4, this was actually the case. A more detailed analysis, however, revealed that, although the IgG detected in the vast majority of patients recognized both oxLDL and MDA-LDL (Fig. 4A), only a small fraction recognized both oxLDL and MDA-HSA (Fig. 4B). On the other hand, the detected IgM that recognized oxLDL also recognized MDA-derivatized LDL and HSA (Fig. 4C, D). Conversely, only a certain fraction of IgM reacting with MDA-derivatized LDL and HSA recognized oxLDL equally well (Fig. 4C, D).

Specificity of oxidatively-modified LDL as the molecule triggering the immune response

The presence of autoantibodies reacting with both MDA-LDL and MDA-HSA in plasma of CRF patients stimulates the question of which molecule is triggering the immune reaction *in vivo*. In other words, the production of autoantibodies could occur in response to either specific LDL oxidation or non-specific oxidative modifications of a variety of other molecules, including HSA. A useful parameter to discriminate between these two possibilities could be the ratio between the antibody titer against MDA-LDL

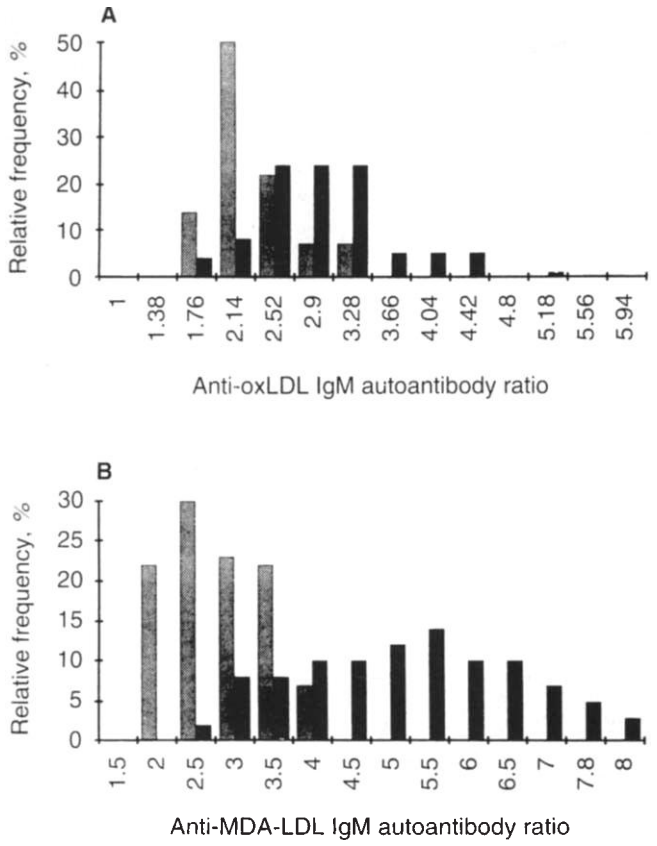

Fig. 2. *Frequency distribution profile of anti-oxLDL and MDA-LDL IgM autoantibodies in control subjects (dashed bars) and CRF patients (black bars).*

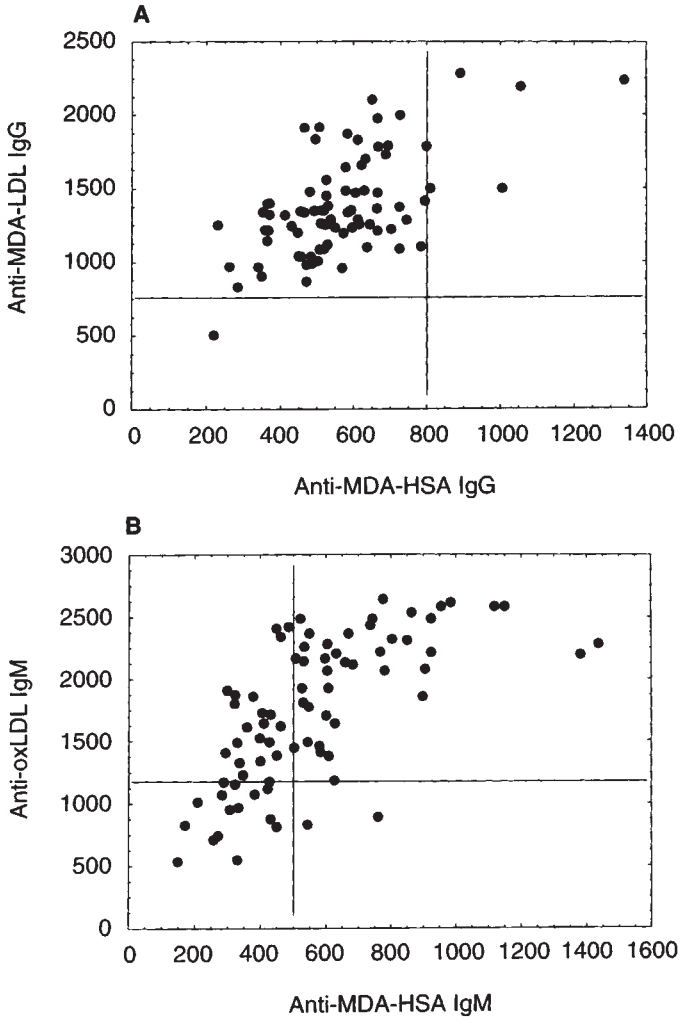

Fig. 3. *Correlation between anti-MDA-LDL and anti-MDA-HSA IgG and IgM autoantibodies in CRF patients.* The spectrophotometric readings (Abs $\times 10^3$) of anti-MDA-LDL IgG (**A**) and IgM (**B**) autoantibodies are plotted against those of anti-MDA-HSA IgG and IgM. The correlation coefficients were $r = 0.599$ ($P < 0.001$) and $r = 0.660$ ($P < 0.001$), respectively. The two lines that form a box in the lower left corner identify the distribution of values from control subjects.

and MDA-HSA. As reported in Table 6, this value was higher than one for both IgG and IgM, indicating that MDA-LDL was preferentially recognized by the antibodies detected in CRF patients. A further analysis revealed a significant difference between CRF patients on hemodialysis as compared to peritoneal dialysis indicating that hemodialysis was, indeed, a more powerful stimulus to specifically induce the production of anti-MDA-LDL autoantibodies.

Detection of immune complexes containing oxLDL in plasma of CRF patients

Eight CRF patients had detectable levels of circulating immune complexes containing oxLDL and IgG. Of the positive patients, six were on hemodialysis (10.7%) and two were on peritoneal dialysis (8.3%). On the other hand, immune complexes containing IgM were undetectable in both groups of patients.

Correlation between anti-oxidatively-modified LDL autoantibodies and clinical setting of CRF patients

No correlation was found between anti-oxidatively-modified LDL autoantibody titer (anti-oxLDL/natLDL IgG and IgM ratio, anti-MDA-LDL/natLDL IgG and IgM ratio, anti-MDA-HSA/HSA IgG and IgM ratio) and any of the following parameters: presence of hypertension, diabetes mellitus, dislipidemia, obesity, duration of the dialytic treatment. In addition, no correlation was found with a score resulting from the clinical evaluation of the severity and extent of atherosclerotic lesions. The clinical score derived from the presence of previous myocardial infarction, angina pectoris, chronic ischemic cardiomyopathy, cerebral chronic vasculopathy and peripheral artery occlusive diseases. Furthermore, no correlation was detected with the concomitant therapy (calcium-channel blocking drugs, cholesterol-lowering drugs, beta-blockers, erythropoietin, inhibitors of the angiotensin converting enzyme).

Discussion

The results obtained in this study demonstrate that: (i) autoantibodies of the IgG and IgM type against oxidatively-modified LDL are produced by CRF patients on dialytic treatment, (ii) the autoantibodies recognize other MDA-derivatized proteins and (iii) immune complexes containing oxLDL are present in approximately 10% of the patients. The detection of circulating autoantibodies against oxidized LDL is considered to be a biological signature of *in vivo* LDL oxidation. This implies that following

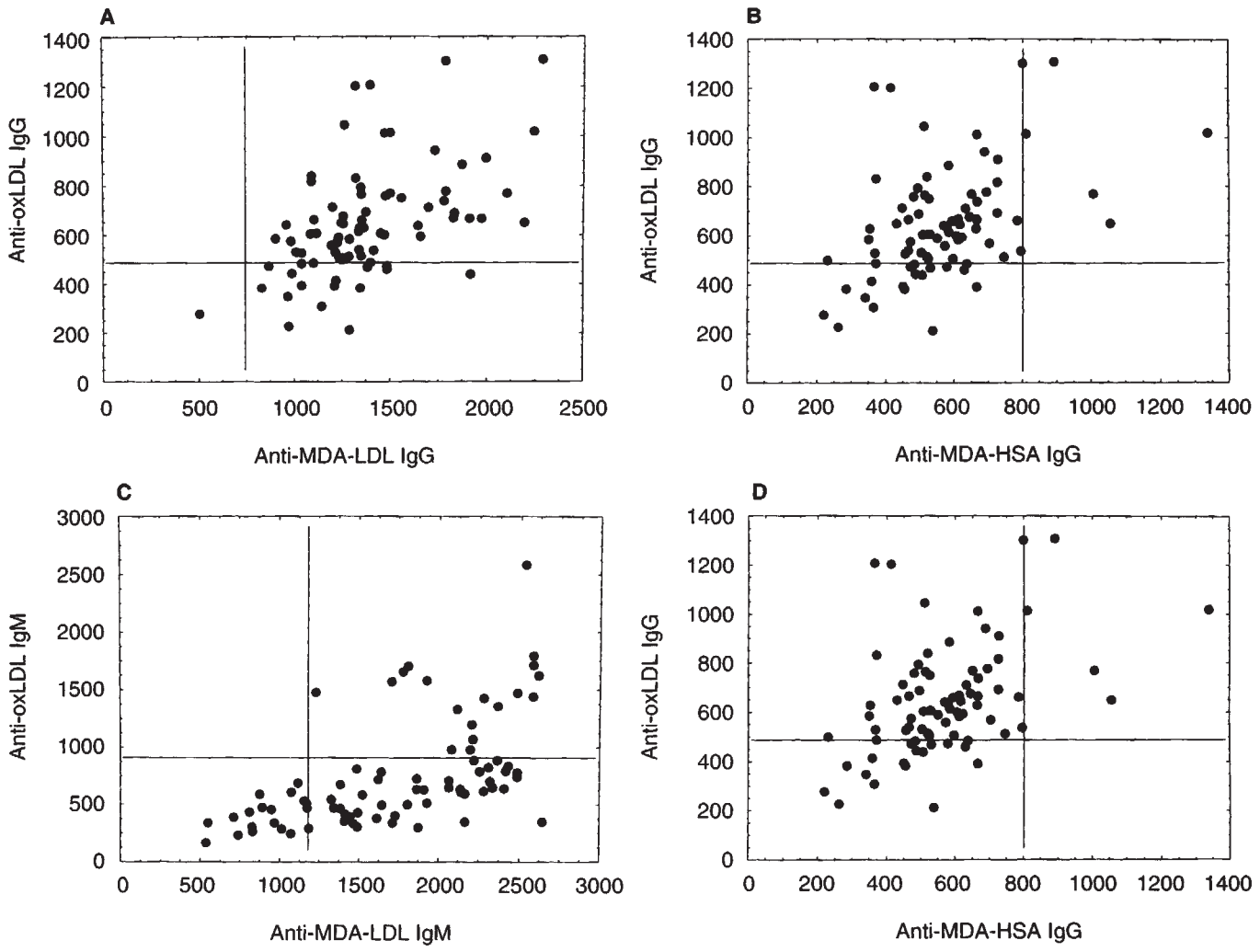

Fig. 4. *Correlation between anti-MDA-LDL and MDA-HSA IgG and IgM with anti-oxLDL IgG and IgM autoantibodies in CRF patients.* The spectrophotometric readings (Abs × 10^3) of anti-MDA-LDL IgG (**A**), anti-MDA-HSA IgG (**B**), anti-MDA-LDL IgM (**C**) and anti-MDA-HSA IgM (**D**) autoantibodies are plotted against those of anti-oxLDL IgG (A and B) and IgM (C and D). The correlation coefficients were $r = 0.52$ ($P < 0.001$), $r = 0.43$ ($P < 0.01$), $r = 0.56$ ($P < 0.001$) and $r = 0.58$ ($P < 0.001$), respectively. The two lines that form a box in the lower left corner identify the distribution of values from control subjects.

Table 6. Specificity of anti-oxidized LDL autoantibodies in plasma of CRF patients on hemodialysis and peritoneal dialysis

Patients	Specific ratio for IgG	Specific ratio for IgM
Total uremic patients ($N = 80$)	1.28 ± 0.41	2.21 ± 1.14
Patients on hemodialysis ($N = 56$)	1.34 ± 0.43	2.41 ± 1.22
Patients on peritoneal dialysis ($N = 24$)	1.12 ± 0.29[a]	1.75 ± 0.78[a]

[a] Statistically different from uremic patients on hemodialysis, $P < 0.05$

oxidative modifications of LDL occurring primarily in the subendothelial space, antigenic epitopes would be generated and would elicit an immune response. Among the various modifications of apoprotein B100 structure occurring during peroxidation of LDL lipids, the derivatization of the ε-amino group of lysine with malondialdehyde and 4-hydroxynonenal is able to stimulate the production of specific antibodies [23-27]. Monoclonal antibodies against oxidized LDL recognize modified apoproteins B100 in early and advanced atherosclerotic lesions in rabbit and in humans [28–31]. Interestingly, in fatty streaks, rich in macrophages, all immunostaining was located intracellularly and some immunostained material was detected extracellularly only in advanced lesions [32]. The detection of autoantibodies of the IgG and IgM type against MDA-modified LDL in CRF patients undergoing dialysis strongly supports the view that this condition is associated with an enhanced production of MDA-LDL occurring *in vivo*. Since MDA represents one of the ultimate products of lipid oxidation, it can conceivably be concluded that the occurrence of this type of autoantibodies may be linked to an enhanced LDL oxidation. Furthermore, the presence of autoantibodies against MDA-modified HSA confirms that the immunogenic determinant is probably the adduct MDA-lysine [23], but causes the non-trivial question of which MDA-modified protein is the real immunogen produced *in vivo* in CRF patients.

An increased level of plasma malondialdehyde, reflecting an enhanced lipid peroxidation, has been reported in uremic patients undergoing hemodialysis [18]. This has been claimed to result from the bioincompatibility of the dialyzer membrane that would

activate polymorphonuclear leukocytes, leading to the generation of large amounts of oxygen activated species that would, in turn, initiate peroxidation of polyunsaturated fatty acids [33]. Concomitantly, a decrease in intracellular and extracellular antioxidant activity would render the target lipids more susceptible to the oxidative attack [18, 34–36]. Under these conditions, a variety of plasma proteins could interact with MDA with the formation of a number of derivatized lysine residues eliciting an immune response. In other terms, the specificity of MDA-derivatized LDL as the immmune trigger would be at least questionable.

The assumption that in the presence of high amounts of MDA, every derivatized protein could stimulate the production of autoantibodies reacting with the MDA-lysine adduct, seems, however, too simplistic. In a recent study we investigated the anti-oxLDL autoantibody titer in three groups of patients with (a) overt coronary atherosclerosis, (b) no clinical signs of atherosclerosis but with an increased cardiovascular risk, and (c) no clinical signs of atherosclerosis but with chronic alcoholic liver disease [8]. The latter group was characterized by a marked increase in plasma peroxide and MDA levels and by the presence of circulating MDA-HSA adducts [37]. The anti-oxLDL autoantibody titer in this group was lower than that detected in atherosclerotic patients and did not differ from that found in control subjects, thus indicating that MDA overproduction was a condition not sufficient to cause the generation of anti-oxLDL autoantibodies.

Another tempting way to escape this impasse has been employed in this work, using the ratio between anti-MDA-LDL/anti-MDA-HSA antibody titer. This approach led to the demonstration that CRF patients had a ratio higher than 1 regarding both IgG and IgM autoantibodies, and that the ratio was lower in CRF patients undergoing peritoneal dialysis as compared to hemodialysis. This implies that: (i) MDA-LDL is the actual immunogen, and (ii) it is preferentially generated in CRF patients on hemodialysis. Support to these conclusions is given by previous reports concerning an increased susceptibility to oxidation of LDL from CRF patients, and the demonstration that short-term peritoneal dialysis increased vitamin E concentration in LDL and ameliorated their resistance to oxidation [17].

The detection of anti-oxidatively modified LDL autoantibodies in CRF patients, in addition to proving the occurrence of LDL oxidation *in vivo*, questions the possible participation of this immune response in the overall process of atherogenesis. The autoimmune hypothesis of atherosclerosis was formulated some 30 years ago and it has been subsequently developed on the basis of new immunological and clinical findings [38, 39]. A particular aspect of this hypothesis predicts that immune complexes containing LDL would be efficiently taken up by macrophages forming foam cells and early atherosclerotic lesions [40]. A significant correlation has been, in fact, observed between the level of circulating immune complexes and the degree of angiographically-demonstrated coronary stenosis [41]. Moreover, selective removal of circulating immune complexes decreased the atherogenic potential of the plasma and improved the clinical setting of the atherosclerotic disease [42]. In order for immune complexes to be formed, a generation of anti-LDL autoantibodies must occur, preceded by a modification of the Apo B100 molecule to make it immunogenic. Three major modifications occurring *in vivo* have been reported to affect the structure and function of LDL: glycation [43], desialylation [44] and oxidation [11]. Among them, only oxidation has been compellingly demostrated to be able to modify the antigenic properties of apoprotein B100 and to stimulate the production of autoantibodies [23, 24]. The results reported here indicate that immune complexes containing oxLDL are present in the plasma of some CRF patients, although with no significant correlation with the type and duration of dialytic treatment and the degree and severity of the atherosclerotic complications.

The detection of immune complexes containing oxLDL implies the presence of oxLDL in the blood stream. Avogaro, Bittolo-Bon and Cazzolato [45] have, indeed, reported that a small fraction of circulating LDL (LDL−) has some of the biochemical features of oxLDL. This view is apparently in contrast with a widely accepted theory that the most important site for LDL oxidation is the subendothelial space [10]. However, it is not possible to exclude that oxLDL present in immune complexes may be generated from the ulceration or rupture of complicated, advanced atherosclerotic plaques, where oxLDL were detected in the extracellular space [31].

The absence of a significant correlation between anti-oxidatively-modified LDL and the degree and extension of the atherosclerotic disease among CRF patients is not surprising. In fact it must be kept in mind that atherosclerosis is a multifactorial disease and LDL oxidation is just a part of it [1]. Moreover, the enhanced LDL oxidation mirrored by high titers of anti-oxLDL autoantibodies seems to be strictly associated with CRF [17, this study]. In other words, it can be considered a factor independent from the other classical risk factors for the progression of atherosclerosis, such as hypertension, hypercholesterolemia, etc.

In conclusion, the findings reported in this work provide a compelling demonstration that anti-oxidatively-modified LDL autoantibodies are present in plasma of CRF patients on dialytic treatment. This demonstration, in addition to proving an enhanced LDL oxidation among uremic patients, envisages a possible participation of oxLDL-triggered immune reactions in the progression of atherosclerosis among these patients. Long-term longitudinal studies to evaluate this possibility are needed and are currently in progress.

Acknowledgments

This work has been supported by grants from Ministero dell'Universitá e della Ricerca Scientifica e Tecnologica, and from IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Policlinico S. Matteo, Pavia, Italy.

Reprint requests to Dr. Giorgio Bellomo, Clinica Medica I, Policlinico S. Matteo, 27100 Pavia, Italy.

Appendix 1. Abbreviations

CRF: chronic renal failure
CS: control subjects
HNE: 4-OH-nonenal
HSA: human serum albumin
LDL: low-density lipoprotein
MDA: malondialdehyde
oxLDL: oxidatively-modified LDL
oxLDLAb: autoantibodies against oxidatively-modified LDL

References

1. Ross R: The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* 362:801–809, 1993
2. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK: Regional accumulations of T-cells, macrophages and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis* 6:131–138, 1986

3. GOWN AM, TSUKADA T, ROSS R: Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human lesions. *Am J Pathol* 125:191–207, 1986
4. OREKHOV AN: Lipoprotein immune complexes and their role in atherogenesis. *Curr Opin Lipidol* 2:329–333, 1991
5. XU Q, DIETRICH H, STEINER HJ, GOWN AM, SCHOEL B, MIKUZ G, KAUFMANN SHE, WICK G: Induction of arteriosclerosis in normocholesterolemic rabbits by immunisation with heat shock protein 65. *Arterioscler Thromb* 12:789–799, 1992
6. XU Q, WILLEIT J, MAROSI M, KLEINDIENST R, OBERHOLLENZER F, KIECHL S, STULLIG T, LUEF G, WICK G: Association of serum antibodies to heat shock protein 65 with carotid atherosclerosis. *Lancet* 341:255–259, 1993
7. SALONEN JT, YLA-HERTTUALA S, YAMAMOTO R, BUTLER S, KORPELA H, SALONEN R, NYYSSONEN K, PALINSKI W, WITZTUM JL: Autoantibodies against oxidized LDL and progression of carotid atherosclerosis. *Lancet* 339:883–887, 1992
8. MAGGI E, FINARDI G, POLI M, BOLLATI P, FILIPPONI M, STEFANO PL, PAOLINI G, GROSSI A, CLOT P, ALBANO E, BELLOMO G: Specificity of autoantibodies against oxidized LDL as an additional marker for atherosclerotic risk. *Coron Art Dis* 4:1119–1122, 1993
9. MAGGI E, MARCHESI E, RAVETTA V, FALASCHI F, FINARDI G, BELLOMO G: Low density lipoprotein oxidation in essential hypertension. *J Hypertens* 11:1103–1111, 1993
10. STEINBERG D, PARTHASARATHY S, CAREW TE, KHOO JC, WITZTUM JL: Beyond cholesterol: Modification of low density lipoproteins that increase its atherogenicity. *N Engl J Med* 320:915–924, 1989
11. ESTERBAUER H, GEBICKI J, PUHL H, JURGENS G: The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad Biol Med* 13:341–390, 1992
12. BERLINER JA, TERRITO M, SEVANIAN A, RAMIN S, KIM JA, RAMSHAD B, ESTERSON M, FOGELMAN AM: Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J Clin Invest* 85:1260–1266, 1990
13. FOGELMAN AM, SHECHTER I, SAEGER J, HOKOM M, CHILD JS, EDWARDS PA: Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci USA* 77:2214–2218, 1980
14. MOREL DW, HESSLER JR, CHISOLM GM: Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipids. *J Lipid Res* 24:1070–1076, 1983
15. LOSOWSKI MS, KENWARD DH: Lipid metabolism in acute and chronic renal failure. *J Lab Clin Med* 71:736–745, 1968
16. LOWRIE E, LAZARUS M, MOCELIN A, BAILEY G, HAMPERS C, WILSON R, MERRIL J: Survival of patients undergoing chronic hemodialysis and renal transplantation. *N Engl J Med* 288:863–868, 1973
17. MAGGI E, BELLAZZI R, FALASCHI F, FRATTONI A, PERANI G, FINARDI G, GAZO A, ROMANINI L, BELLOMO G: Enhanced LDL oxidation in uremic patients: An additional mechanism for accelerated atherosclerosis? *Kidney Int* 45:876–883, 1994
18. TOBOREK M, WASIK T, DROZDZ M, KLIN M, MAGNER-WROBEL K, KOPIECZNA-GRZEBIENIAK E: Effect of hemodialysis on lipid peroxidation and antioxidant system in patients with chronic renal failure. *Metabolism* 41:1229–1232, 1992
19. GIRELLI D, OLIVIERI O, STANZIAL AM, AZZINI M, LUPO A, BERNICH P, MENINI C, GAMMARO L, CORROCHER R: Low platelet glutathione peroxidase activity and serum selenium concentration in patients with chronic renal failure: Relations to dialysis treatments, diet and cardiovascular complications. *Clin Sci* 84:611–617, 1993
20. GOTCH FA, SARGENT JA: A mechanistic analysis of the National Cooperative Dialysis Study (NCDS). *Kidney Int* 28:526–534, 1985
21. ESTERBAUER H, STRIEGL G, PUHL H, ROTHENEDER M: Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Rad Res Commun* 6:67–75, 1989
22. JURGENS G, HOFF HF, CHISOLM GM, ESTERBAUER H: Modification of human serum low density lipoprotein by oxidation: characterization and pathophysiological implications. *Chem Phys Lipids* 45:315–336, 1987
23. PALINSKI W, YLA-HERTTUALA S, ROSENFELD ME, BUTLER S, SOCHER SA, PARTHASARATHY S, CURTISS LK, WITZTUM JL: Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis* 10:325–335, 1990
24. CHEN Q, ESTERBAUER H, JURGENS G: Studies on epitopes on low-density lipoprotein modified by 4-hydroxynonenal. *Biochem J* 288: 249–254, 1992
25. STEINBRECHER UP: Oxidation of human low density lipoprotein results in derivatisation of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J Biol Chem* 262:3603–3608, 1987
26. STEINBRECHER UP, FISHER M, WITZTUM JL, CURTISS LK: Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation or carbamylation: generation of antibodies specific for derivatised lysine. *J Lipid Res* 25:1109–1116, 1984
27. GOONEN B, FALLON JJ, BACKER SA: Immunogenicity of malondialdehyde-modified low density lipoproteins. Atherosclerosis 65:265–272, 1987
28. PALINSKI W, ROSENFELD ME, YLA-HERTTUALA S, GURTNER GC, SOCHER SS, BUTLER S, PARTHASARATHY S, CAREW TE, STEINBERG D, WITZTUM JL: Low density lipoprotein undergoes oxidative modification *in vivo*. *Proc Natl Acad Sci USA* 86:1372–1376, 1989
29. YLA-HERTTUALA S, PALINSKI W, ROSENFELD ME, PARTHASARATHY S, CAREW TE, BUTLER S, WITZTUM JL, STEINBERG D: Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 84:1086–1095, 1989
30. MOWRI H, OHKUMA S, TAKANO T: Monoclonal DLRIa/104G antibody recognizing peroxidized lipoproteins in atherosclerotic lesions. *Biochim Biophys Acta* 963:208–214, 1988
31. ROSENFELD ME, PALINSKI W, YLA-HERTTUALA S, BUTLER S, WITZTUM JL: Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbit. *Arteriosclerosis* 10:336–349, 1990
32. BOYD HC, GOWN AM, WOLFBAUER G, CHAIT A: Direct evidence for a protein recognized by a monoclonal antibody against oxidatively-modified LDL in atherosclerotic lesions from a Watanabe heritable hyperlipidemic rabbit. *Am J Pathol* 135:815–825, 1989
33. GRISHAM MB, MCCORD JM: Chemistry and cytotoxicity of reactive oxygen metabolites, in *Physyiology of Oxygen Radicals*, Baltimore, American Physiological Society 1986, pp 1–18
34. KURODA M, ASAKA S, TOKOFU Y, TAKEDA R: Serum antioxidant activity in uremic patients. *Nephron* 41:293–298, 1985
35. THEIL GB, BRODINE GE, DOOLAN PD: Red cell glutathione content and stability in renal insufficiency. *J Lab Clin Med* 58:736–742, 1961
36. RICHERD MJ, ARNAUD J, JURKOVITZ C, HACHACHE T, MEFTAHI H, LAPORTE F, FORET M, FAVIER A, CORDONNIER D: Trace elements and lipid peroxidation abnormalities in patients with chronic renal failure. *Nephron* 57:10–15, 1991
37. CLOT P, TABONE M, ARICÒ S, DIANZANI MU, ALBANO E: Biological markers of oxidative damage in patients with alcoholic cirrosis: Evidence for a specific relationship with alcohol intake. *Alcohol & Alcoholism* 28:237, 1993
38. BEAUMONT JL: Immunological factors in atherosclerosis, in *Handbook of Disease of Aging,* edited by BLUMENTHAL HT, New York, Van Nostrand, 1983, pp 317–336
39. LOPEZ-VIRELLA MF, VIRELLA G: Immune mechanisms in the pathogenesis of atherosclerosis. *Adv Exp Med Biol* 285:383–392, 1991
40. KLIMOV AN, DENISENKO AD, VINOGRADOV AG, NAGORNEV VA, PIVOVAROVA YI, SITNIKOVA OD, PLESKOV VM: Accumulation of cholesteryl esters in macrophages incubated with human lipoprotein-antibody autoimmune complex. *Atherosclerosis* 82:227–235, 1990
41. TERTOV VV, OREKHOV AN, KACHARAVA AG, SOBENIN IA, PEROVA NV, SMIRNOV VN: Low density lipoprotein-containing circulating immune complexes and coronary atherosclerosis. *Exp Mol Pathol* 52:300–308, 1990
42. CHAZOV EI, OREKHOV AN, TERTOV VV, POKROVSKY SN, ADAMOVA IY: Atherogenicity of blood plasma from patients with coronary atherosclerosis and its correction. *Atherosclerosis Rev* 17:9–20, 1988
43. LYONS TJ, KLEIN RI, BAYNES JW, STEVENSON HC, LOPEZ-VIRELLA MF: Stimulation of cholesteryl ester synthesis in human monocyte-derived macrophages by low-density lipoproteins from type-1 (insulin-dependent) diabetic patients: The influence of non-enzymatic glycosylation of low density lipoproteins. *Diabetologia* 30:916–923, 1987
44. OREKHOV AN, TERTOV VV, MUKHIN DN: Desialylated low density lipoprotein: Naturally occurring modified lipoprotein with atherogenic potency. *Atherosclerosis* 86:153–161, 1991
45. AVOGARO P, BITTOLO-BON G, CAZZOLATO G: Presence of a modified low density lipoprotein in humans. *Atherosclerosis* 8:79–87, 1988